NMR and circular dichroism studies of synthetic peptides derived from the third intracellular loop of the β-adrenoceptor

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Abstract The C-terminal part of the third intracellular loop of the β-adrenoceptor is capable of stimulating adenylate cyclase in the presence of phospholipid vesicles via the stimulatory guanine nucleotide binding protein (G_s) [Palm et al. (1989) FEBS Lett. 254, 89–93]. We have investigated the structure of synthetic peptides corresponding to residues 284–295 of the turkey erythrocyte adrenoceptor in micelles, trifluoroethanol and aqueous solution, by using 2D ^{1}H NMR and CD. In the presence of phospholipid micelles the peptides display a C-terminal α-helical region, whereas the N-terminal part was found to be highly flexible.

Key words: β-Adrenoceptor; Circular dichroism; Conformational change; ^{1}H NMR; Peptide–phospholipid interaction

1. Introduction

Protein–protein interactions play a pivotal role in transmembrane signalling by G protein-coupled receptors. The ability of the receptor to bind to the intracellular G protein after extracellular stimulation and to set off the GDP/GTP exchange and GTPase activity is essential for the activation cascade. To understand how the signal is transferred to, and recognized by, the corresponding G protein will require more information on the tertiary structure of the intracellular loops of the receptor.

It was proposed that upon binding of the hormone to the extracellular site of the receptor, the intracellular loops adopt a conformation which favors their interaction with the mediating G protein [1]. Alternatively it was suggested that agonist binding will expose prototypic amphiphilic α-helical structures at an intracellular site for specific interaction with G proteins [2,3]. If the functional properties of these sites can be represented by peptides, those peptides should also serve as a model for the structural features of the corresponding receptor regions.

Peptides representing parts of the intracellular loops of the β-adrenergic receptor from turkey erythrocytes were synthesized. Their effects on hormonal stimulation of adenylate cyclase was measured in isolated membranes or by incubation of individual components of the β-adrenergic adenylate cyclase system which were functionally reconstituted in phospholipid vesicles [4,5]. While most peptides only decouple hormonally stimulated adenylate cyclase by competing with the corresponding receptor sites, the peptide T284-295 with the sequence REHKALKTLGII, comprising the C-terminal part of the third intracellular loop and extending into transmembrane helix 6, was the first receptor-derived peptide that was able to stimulate adenylate cyclase in a G protein-dependent manner. Moreover, this peptide can replace stimulatory receptors, like the β-adrenergic receptor, in partially reconstituted phospholipid vesicles containing only G_s and adenylate cyclase. We proposed that this peptide assumes the active conformation of the corresponding receptor region.

For a better understanding of the molecular basis of signal transduction it is therefore desirable to uncover structural features of peptides involved in receptor coupling. Circular dichroism and NMR spectroscopy were used to monitor the native state and structural transitions of those peptides.

2. Materials and methods

2.1. Materials

DIC and HOBT were obtained from Fluka, Nucleosil columns from Macherey & Nagel. Protected amino acid derivatives were purchased from Calbiochem-Novabiochem, myristoyl-lyso-PC from Sigma, and deuterated myristoyl-lyso-PC from Avanti Polar Lipids. TFE-d_3 was obtained from Cambridge Isotopes.

2.2. Peptide synthesis and analytical procedure

Peptides were synthesized as peptide amides using a Tentagel RAM resin on a Zinsser Analytic SMPS 350 A peptide synthesizer. Solid-phase syntheses were performed with 9-fluorenylmethylcarbonyl (Fmoc) for protection and with DIC and HOBT for activation chemistry. A mixture of TFA/anisole/ethanedithiol was used for cleavage. The peptides were purified by reverse-phase HPLC on a Nucleosil 300–7 C18 (20 × 250 mm) column using a H_2O/0.1% TFA-acetonitrile gradient. The calculated mass of the sequences was confirmed by MALDI-TOF mass spectroscopy.

2.3. Circular dichroism

CD spectra were recorded on a Jobin Ivon CD6 spectrometer. Ellipticity is reported as mean residue molar ellipticity. For preparing micelles the lyso-PC-water mixture was homogenized using a vortex mixer.

2.4. Nuclear magnetic resonance

Different amounts of TFE-d_3 or deuterated lyso-PC were added to peptide samples at pH 4.15 and 10% D_2O in 5 mm sample tubes. NMR was performed on Bruker AM300 and AMX500 NMR spectrometers, operating at 300 MHz and 500 MHz proton frequency. In 2D experiments [6,7], quadrature detection in F1 dimension was achieved analogous to the method of Redfield [8], using time proportional phase

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Abbreviations: 2D, 3D, two-, three-dimensional; CD, circular dichroism; COSY, correlated spectroscopy; DIC, diisopropylcarbodiimide; G-protein, guanine nucleotide binding regulatory protein; G_s protein, stimulatory G protein; G_o protein, a G protein from brain; HOBT, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption/ionisation-time of flight; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; lyso-PC, lyso-phosphatidylcholine; ROESY, rotating frame Overhauser enhancement spectroscopy; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TFE-d_3, perdeuterated trifluoroethanol; TOCSY, total correlation spectroscopy; TSP, 3-trimethylsilyltetradeuteropropionate.

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increments (TPPI) [9]. The water signal was suppressed by a presaturation pulse, typically 2.5 s long. In addition, the water resonance was saturated during mixing in NOESY experiments [10–12]. COSY [13–15] ROESY [16,17] and TOCSY [18,19] experiments were performed according to the standard pulse schemes. Chemical shifts are expressed downfield to the methyl group of TSP.

3. Results

3.1. Circular dichroism measurements

The CD spectra of the peptides T284-294 and T284-295 were obtained in aqueous solution, TFE and in the presence of micelles. The spectra of both peptides never differed, when compared under the same experimental conditions. Therefore, only the spectra of T284-295 are presented. Fig. 1 shows the effect of 10 mM lyso-PC on the CD spectra of T284-295. The structure of the peptide changes from random chain (open diamonds), with its typical minimum at 199 nm, to a peptide with 50% α-helix (crosses), according to quantifications of Greenfield and Fasman [20]. Fig. 2 demonstrates the results with various concentrations of TFE. An isodichroic point was observed at 203 nm, indicating that the interchange between α-helix and random chain in the presence of TFE can be described as a two state process [21,22]. At a concentration of 64% TFE 40% α-helix was detected, whereas at 95% TFE, 70% of the peptide shows an α-helical conformation with the typical minima at 207 and 223 nm. Hence, high concentrations of TFE are required to bring about a conformation of high α-helicity to this peptide.

3.2. Nuclear magnetic resonance

Both peptides T284-294 and T284-295 were first dissolved in 90% TFE-d_3. ROESY, NOESY, TOCSY and COSY experiments indicate an α-helical structure and made a resonance assignment possible which was used as basis for the resonance assignment in the presence of micelles. Measurements of the peptide T284-294 in aqueous solution revealed no structural features, a random chain has to be assumed. In the presence of 60 mM deuterated lyso-PC micelles NOESY and TOCSY experiments were performed on T284-294. Fig. 3 shows the resonances of the backbone amide protons and their assignments. The NOESY experiment reveals 5 cross peaks in the amide region that all were identified as sequential NH_i–NH_{i+1} cross-peaks connecting neighbouring backbone amide protons

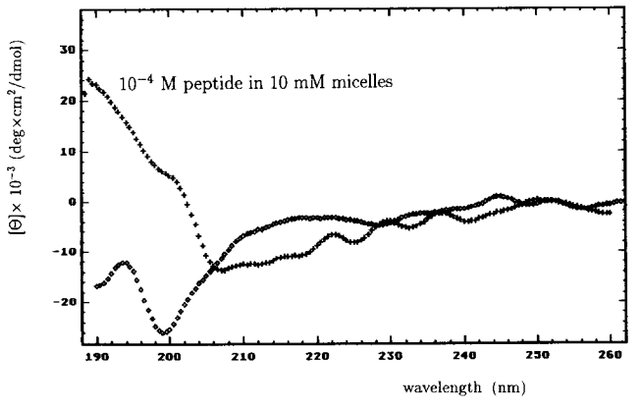

Fig. 1. CD spectra of T284-295 in the absence and presence of 10 mM lyso-PC micelles. The concentration of the peptide was 10^{-4} M at pH 7 and 23°C.

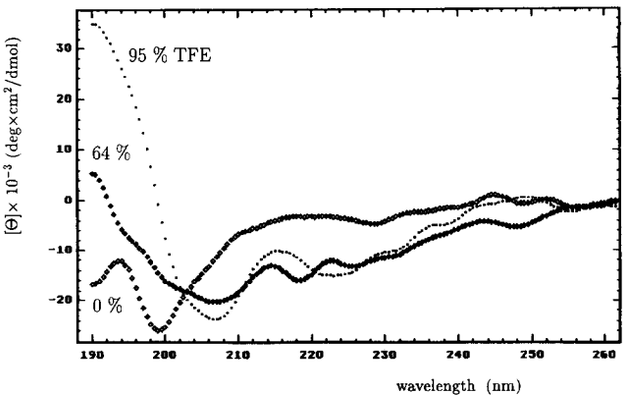

Fig. 2. CD spectra of T284-295 in the presence of TFE. Concentrations of TFE were 0% (open diamonds), 64% (filled diamonds) and 95% (dots). All other parameters are the same as indicated in Fig. 1.

from the core of the peptide to those at the C-terminal end (Fig. 4). In NOESY experiments magnetization is only transferred over short distances, roughly the intensity of the cross peak is proportional to $1/r^6$, with r being the distance [23]. Since the distances between neighbouring backbone amide protons in α-helices are small the observed NH_i–NH_{i+1} correlations indicate an α-helical structure [24].

4. Discussion

Circular dichroism and high resolution proton NMR spectroscopy have been shown to be sensitive techniques to monitor structural properties and structural transitions of peptides in environments as different as polar solvent and phospholipid micelles.

Low concentrations of TFE can induce an α-helical conformation in short peptides which were characterized by CD [21]. T284-294 and T284-295 reveal a rather low tendency to adopt an α-helical structure even at high concentrations of TFE. Similar results were obtained in 10 mM lyso-PC which lead to 50% α-helix. In contrast to T284-295, the wasp venom mastoparan which directly interacts with G proteins shows a complete α-helix in the presence of 10 mM lyso-PC [25]. We propose from the CD spectra that the helical part of the receptor derived peptide T284-295 is probably associated with the phospholipid micelles, whereas the rest of the peptide is flexible like a random chain.

2D ^{1}H NMR spectroscopy makes it possible, however, to determine those parts of the peptide T284-294 which become highly structured upon binding to micelles. 2D experiments of T284-294 reveal that one turn of an α-helix extending from Lys-290 to the C-terminal Ile-294 is formed in the presence of phospholipid micelles. There is also a close proximity between the backbone amide protons of Ala-288 and Leu-289, but the corresponding cross-peak of Leu-289 to Lys-290 is absent. This may indicate a smooth changeover from the helix in the C-terminal part of the peptide to a random chain in the N-terminal part of the peptide. The results also show that the C-terminal Ile-295 can be omitted. Therefore, either peptide T284-294 or T284-295 can be used to elucidate the structure of T284-295 in the presence of phospholipid micelles and it can be expected that the secondary structure asssignments of the

synthetic peptide match closely the properties of the corresponding receptor region.

The assignment of a C-terminal helix extending from residue 290 to 295 of the peptide agrees favorably with the predicted extension of the sixth transmembrane helix distal to residue 291 [26]. From the assignment of Lys-290 to the helical part this residue can be considered either as an integral part of the membrane or as part of a cytoplasmic extension of the transmembrane helix [2,27].

In contrast, the N-terminal part lacks defined structures within the limits of detection, which led us to assume that minor secondary structure constraints allow a high level of flexibility. In accordance with the flexibility of the peptide the same region in the receptor might easily switch from the inactive to the active state of the receptor or assume a complementary structure to the presumed recognition site of the G protein by induced fit. However, it has to be kept in mind that the 'open end' of the small peptide might be more constrained in the corresponding receptor environment.

The receptor-derived synthetic peptide and the liganded receptor behave in a functionally analogous manner in activating G_s protein [4,5]. In addition, in this study structural similarities have been established. Therefore, we propose that this peptide serves as an appropriate model to describe the essential structural and functional features of the corresponding receptor site.

In the past, mastoparan was considered to be the prime example to model G protein-linked receptor coupling [28]. It was shown that mastoparan adopts almost exclusively a single conformation in phospholipid vesicles or when in fast exchange with G_o protein [29,30]. In contrast to mastoparan, site-specific, receptor-derived peptides, including T284-294, seem to be endowed with discrete domains one of which is conformationally flexible. It is tempting to speculate that the receptor shifts from an unliganded, inactive to a liganded, active state by translocation of the sixth transmembrane helix concomitant with exposure of a hydrophobic helical fragment to the surface. This portion together with the flexible part could serve as a counterpart to the bipartite receptor recognition site on the G protein. The composite nature of this binding site is supported by the very recent report that an α_2-adrenoceptor-derived peptide can

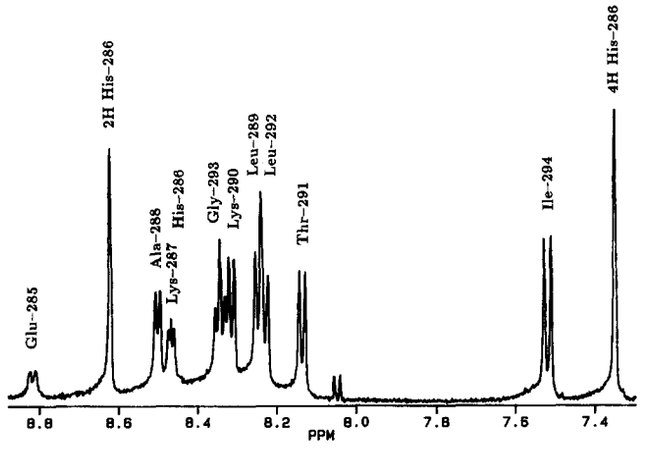

Fig. 3. NH region of a 500 MHz ^{1}H NMR spectrum of T284-294 (5 mM) in the presence of 60 mM deuterated lyso-PC at pH 4.15 and 30°C. The backbone amide resonances as well as the ring protons of His-286 are indicated.

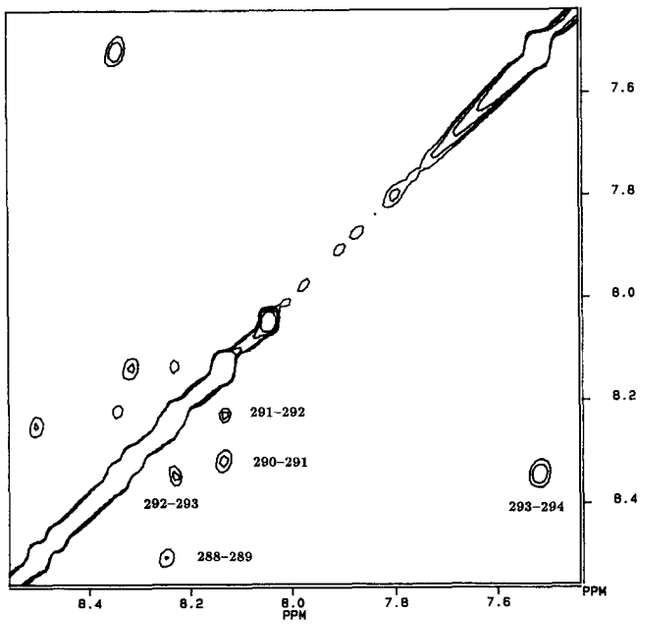

Fig. 4. NH–NH region of a 500 MHz NOESY spectrum of T284-294 (5 mM) in the presence of 60 mM deuterated lyso-PC at pH 4.15 and 30°C. The mixing time was 200 ms. The number located on one side of each cross peak labels the NH–NH correlation of the corresponding amino acid residues.

specifically be cross-linked to both the α- and β-subunits of the corresponding G protein [31]. Mastoparan, on the contrary, can form only cross-links to the αsubunit [32], indicating that mastoparan is able to fulfill only one aspect of the structural requirements for G protein coupling.

For the first time the conformational properties of a receptor-derived G protein-stimulating peptide were determined in the presence of phospholipids. This reveals a rather rigid, membrane-associated conformation joined to an easily adjustable sequence, probably 'linked' to corresponding G proteins by its sequence. Interaction with the corresponding G protein would require nothing else than exposure of the 'peptide region' after receptor activation to the complementary G protein site for binding.

More receptor-derived peptides have to be characterized to extract the structural features required for receptor-specific interaction during coupling.

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